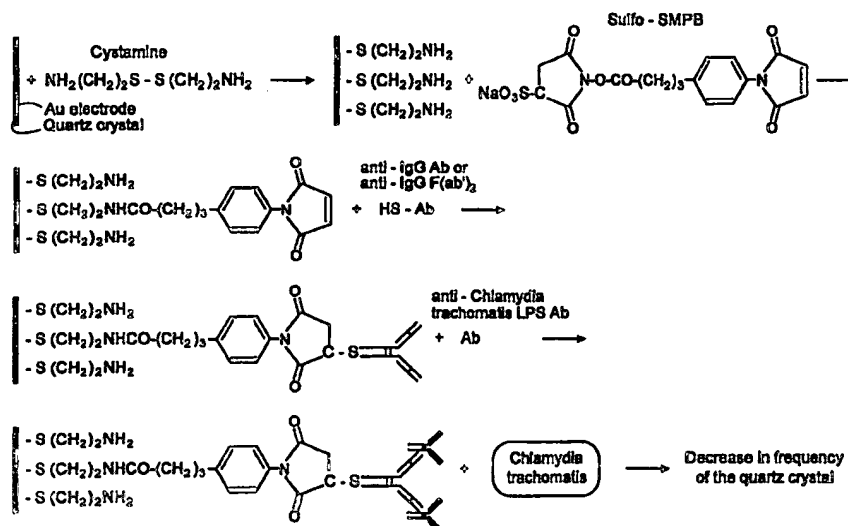




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<p>(21) International Application Number: PCT/IL98/00119</p> <p>(22) International Filing Date: 13 March 1998 (13.03.98)</p> <p>(30) Priority Data: 120445 13 March 1997 (13.03.97) IL</p> <p>(71) Applicant (for all designated States except US): YISSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM [IL/IL]; 46 Jabotinsky Street, 92182 Jerusalem (IL).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): WILLNER, Itamar [IL/IL]; 12 Hashalom Street, 90805 Mevasseret Zion (IL). BEN-DOV, Iddo [IL/IL]; 5 Pri Megadim Street, 90805 Mevasseret Zion (IL). ZISMAN, Einat [IL/IL]; Reuven and Batsheva 1, 75357 Rishon Le Zion (IL). LEVI, Shlomo [IL/IL]; 128 Hapalmach Street, 90805 Mevasseret Zion (IL).</p> <p>(74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel Aviv (IL).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>

(54) Title: BIOSENSOR FOR CELLS



(57) Abstract

A system and method for assaying presence and optionally concentration of cells in a liquid medium, as well as a sensing member for use in the system or method, are provided. The sensing member comprises a piezoelectric crystal provided on its surface with a metal plate in which there are immobilized specific binding entities which specifically bind to an epitope on the surface of said cells. Binding or release of the cells from the specific binding entities causes a change in mass and hence a change in resonance frequency of the piezoelectric crystal. Measuring the change in the resonance frequency provides an indication of presence of the assayed cells in a medium. Determining of the extent of change in resonance frequency may be used to determine the concentration of the cells in the medium. By a specific embodiment the assayed cells are chlamydia and the liquid medium is urine.

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BIOSENSOR FOR CELLS

FIELD OF THE INVENTION

The invention is generally in the field of biosensors, and concerns a sensor useful for the determination of the presence, and optionally concentration, of cells in medium, e.g. in urine, blood or mucous. The present
5 invention relates to such sensors, as well as to their use and systems comprising them.

BACKGROUND OF THE INVENTION AND PRIOR ART

In the following description, reference will be made to several
10 prior art documents shown in the list of references below. The reference will be made by indicating their number from this list.

Chlamydia trachomatis has been acknowledged as a widespread sexually transmitted microorganism (1). In women, *C. trachomatis* infections can lead to infertility, ectopic pregnancy, and chronic
15 peivic pain. In men, *C. trachomatis* can cause urethritis and epididymitis. Newborns to *C. trachomatis* infected mothers are in risk of chlamydial conjunctival and pulmonary disease.

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Cell culture offers a general means for the isolation and identification of *C. trachomatis*. Various immunoassays for *C. trachomatis* were also developed, which include enzyme-linked immunosorbant assays (ELISA) and fluorescence labeled antibody assay. The available assays are either limited in their sensitivity, are time consuming and require skilled personnel or are subjective. Polymerase chain reaction (PCR) was found to be sensitive and specific in testing specimens from men with non-gonococcal urethritis. This method is, however, time-consuming (requires about 12-24 hours). Detection of anti-*C. trachomatis* antibodies is problematic in that antibodies formation lags behind the progress of the infectious state, in that the antibodies may exist in the serum several years after infection, and in that the anti-*Chlamydia pneumoniae* or anti-*Chlamydia psittaci* antibodies may exist in the serum which obscure the results. Such procedures are also expensive and require highly skilled personnel. Thus, the development of a highly-sensitive detection means for *C. trachomatis* in urine specimens or urethral swabs containing urethral mucous, could provide an important diagnostic method for this infection.

The specificity of antigen-antibody binding interactions and the technological progress in the preparation of monoclonal antibodies provide grounds for the design of sensitive immunosensor devices for clinical diagnostics, food control or monitoring of environmental pollutants. The linear relationship between the change in the oscillating frequency of a piezo-electric crystal and the mass variation on the crystal as a result of binding or adsorption enables the efficient monitoring of antigen-antibody binding. The mathematical relation between the change in resonance frequency of a piezoelectric crystal, Δf , and mass changes, following Sauerbrey equation (1):

$$\Delta f = -2.3 \times 10^6 f_0^2 \cdot \Delta m / A \quad (1)$$

where f_0 is the resonance frequency of the crystal prior to the mass variation and A is the surface area of deposited mass. For example, for a crystal exhibiting a fundamental resonance frequency of 9 MHz and surface area of 1
5 cm^2 a mass-change on the crystal that corresponds to 1×10^{-9} g will give rise to a resonance frequency change, Δf , of 6 Hz.

The use of quartz piezoelectric crystals or the so called "quartz crystal microbalance" (QCM) has been adopted for the detection of antigens or antibodies (Ab) in a series of analytical studies. (See review by Suleiman *et*
10 *al.*, 1994 (2)).

Physical adsorption of antigens to a crystal was applied for the detection of antigens by interacting the crystal with a mixture of the analyte antigen and a predetermined amount of Ab (3). The decrease in the antigen concentration was inversely related to the antigen concentration in the sample.
15 Two patents by Rice (4 and 5), describe methods for the determination of Abs by QCM. The antigen was immobilized on a polymer pre-coated crystal and the frequency changes as a result of Ab association related to the analyte Ab concentration in the sample. By this method, human IgG against honey bee venom, phospholipase A, and keyhole limpet hemocyanin were analyzed.
20 Non-specific binding to the crystal interfered with the analyses. Later on the detection of low molecular weight components by a pre-coated crystal with the anti-Ab and competitive binding assay of the Ab-low molecular weight analyte was described. All of these analyses were performed by treatment of the crystals in solution and subsequent frequency measurements in air. This
25 two-step solution/gas procedure allows improvement of the sensitivity of the resonating QCM, but introduces severe drawbacks as it introduces technical complications and the interference of hydration/dehydration phenomena that are reflected in the frequency parameters.

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Piezoelectric immunoassaying in the liquid phase has important technical advantages as it allows stationary and flow analysis of aqueous samples. The method suffers, however, from a basic physical limitation due to substantially lower frequency changes of the crystal as a result of the solution viscosity. QCM immunoassays in solution were disclosed by Roederer (6). The quartz crystal was modified with glycidoxypopyltrimethoxy silane (GOPS), and the surface-modified crystal was then further modified by anti-human IgG antibody and then applied for the piezoelectric detection of human IgG. The detection limit of the device was determined to be 13 $\mu\text{g}\cdot\text{ml}^{-1}$.

QCM has been used also to analyze large entities such as microbial cells, using antibody-coated quartz crystals. *Candida albicans* cells in the concentration range 1×10^6 - 5×10^8 cells $\cdot\text{ml}^{-1}$ were analyzed by an anti-*Candida albicans* coated Ab surface (7), *E. coli* with an anti-*E. coli* coated surface (8) and protein A-coated crystals acted as piezoelectric sensing interface for various bacteria including *Salmonella*, *Shigella*, *Yersinia* and *E. Coli*.

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GENERAL DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a system, method and sensor for determining the presence and optionally the concentration of cells in a liquid medium, specifically but not exclusively in body fluid samples, e.g. urine, serum, plasma, whole blood, cerebro spinal fluid (CSF),
5 amniotic fluid, etc.

The present invention makes use of a piezoelectric crystal coated by entities, e.g. monoclonal antibodies, which specifically bind the assayed cells (such entities will be referred to herein at times as "*specific*
10 *binding entities*"). A change of mass bound to the crystal reflected by a change of resonance frequency of the crystal is indicative of the presence of said cells in the tested medium. In the following, the term " *Δf response*" will be used to denote a change of frequency of the sensor as the result of binding of a mass thereto or release of a mass therefrom.

15 In accordance with the present invention, a novel system and method as well as sensors for use in the system and method are provided. In accordance with the present invention, the " *Δf response*" of the sensor is measured and serves for determination of the presence and optionally the concentration of cells in the assayed liquid medium.

20 In accordance with the first aspect of the invention there is provided a system for assaying cells in a liquid medium, the system comprising a piezoelectric crystal-based sensing member; an electric or electronic unit electrically connectible to the crystal for generating a vibration inducing electric current causing vibrations in said crystal and for measuring its
25 resonance frequency; and a vessel for holding a specimen of said liquid medium and allowing contact thereof with said sensing member; the sensing member comprising a piezoelectric crystal provided with one or more metal plates on its surface, the metal plates having immobilized thereon specific binding entities, which specifically bind to an epitope on the surface of said

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cells, such that the binding of the cells to the immobilized specific binding entities or release of said cells from the immobilized member bringing to a change of mass of the sensing member, resulting in a change of the sensing member's resonance frequency measurable by said unit.

5 The assay is performed by first providing conditions allowing binding of the cells to the specified binding entities. Then the change in resonance frequency following binding may be measured. Alternatively, the cells are first allowed to bind to the specific binding entities and then conditions are provided which cause release of said cells and then the change
10 in frequency following such a release is measured. Regardless of the manner in which the assay is performed, the measured change in resonance frequency serves as an indication for the presence of the assayed cells in the assayed liquid medium and the extent of change serves as an indication of the concentration of the cells in the assayed medium.

15 In accordance with the second aspect of the invention there is provided a method for assaying presence and optionally concentration of cells in a liquid medium, the method comprising:

(a) providing a sensing member comprising a piezoelectric crystal with one or more metal plates on its surface, the metal plates having
20 immobilized thereon specific binding entities which specifically bind to epitopes on the surface of said cells;

(b) contacting said sensing member with a specimen of said medium for a time and under conditions allowing binding of the cells to said specific binding entities;

25 (c) inducing vibrations in said crystal and measuring a change in the resonance frequency as a result of either

(ca) contact of said sensing member with said specimen,

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(cb) contact as in (ca) and then incubation of said sensing member under conditions and for a time such that cells bound to said specific binding entities will be released, or

5 (cc) contact as in (ca) followed by exposure of the sensing member to a sensitivity increasing agent, which is a free agent which specifically binds to an epitope on the surface of said cells; said change being an indication of presence of said cells in said medium.

Optionally, the extent of the change is also monitored and serves as an indication of the concentration of said cells in said medium.

10 In accordance with yet a further aspect of the invention there is provided a sensing member useful in the above system and method.

The specific binding entities, are entities which specifically bind to an epitope on the surface of such cells. They may, for example, be antibodies, particularly monoclonal antibodies, which recognize an epitope
15 presented on the surface of said cells. By another example, said entities are lectins which specifically bind to sugar moieties on the surface of said cells.

Occasionally, the sensing member may contain on its surface some non-specific binding sites which are capable of adsorption of large molecules, e.g. proteins. In order to block such binding sites, following
20 binding of the specific binding entities to the sensing member, macromolecules, e.g. proteins, may be adsorbed to such binding sites. Such blocking may be achieved, for example, by soaking the sensing member with bovine serum albumin (BSA), animal (e.g. fish) sera, gelatin, casein, polymers, e.g. polyethyleneglycol, saccharides, e.g. sucrose, trehalose, etc.

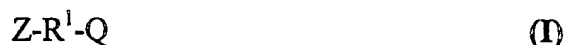
25 In order to cause a piezoelectric crystal to vibrate and eventually reach resonance frequency, the piezoelectric crystal has to be subjected to alternating electrical field. The piezoelectric crystal used in accordance with the invention is typically a planar crystal having electrodes which are made of an electrically conducting material and which typically have the form

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of plates (rectangular, circular, etc.), attached to opposite faces of the crystal. The plates carrying the immobilized binding entities can function also as the electrodes which convey the alternating electric field to the crystal. These plates may be selected from a variety of conducting substances, preferred
5 being such which are made from or which are at least coated by a metal or which can chemisorb a sulphur-containing moiety, e.g. gold, platinum or silver.

The specific binding entities may be immobilized on the metal plate by means of a linking group having the following general formula (I):

10



wherein:

Z represents a sulphur-containing moiety which is capable of
15 chemical association with, attachment to or chemisorption onto said metal;

R¹ represents a connecting group;

Q is a functional group which is capable of forming a covalent bond with a moiety in the specific binding entity.

20

Z, may for example be a sulphur atom obtained from a thiol group, a disulfide group, a sulphonate group, or a sulphate group.

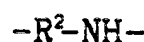
R¹ may be a covalent bond or may be a peptide or polypeptide or may be selected from a very wide variety of suitable groups such as
25 alkylene, alkenylene, alkynylene phenyl containing chains, and many others.

Particular examples of **R¹** are a chemical bond or a group having the following formulae (IIa), (IIb), (IIc) or (IId)

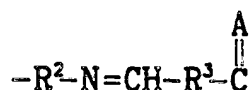
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II (a)



II (b)



II (c)



II (d)

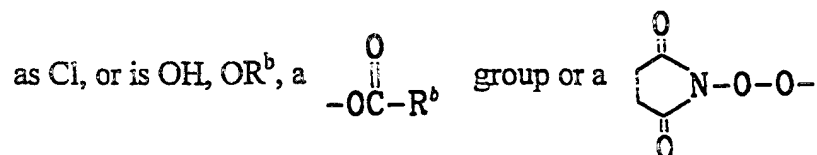
wherein

R^2 or R^3 may be the same or different and represent straight or branch alkylene, alkenylene, alkynylene having 1-16 carbon atoms or represent a covalent bond,

A and B may be the same or different and represent O or S,

Ph is a phenyl group which is optionally substituted, e.g. by one or more members selected from the group consisting of SO_3^- or alkyl groups.

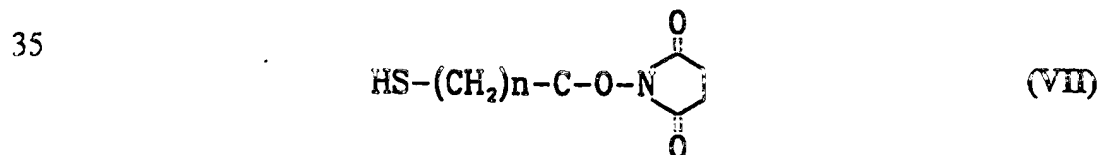
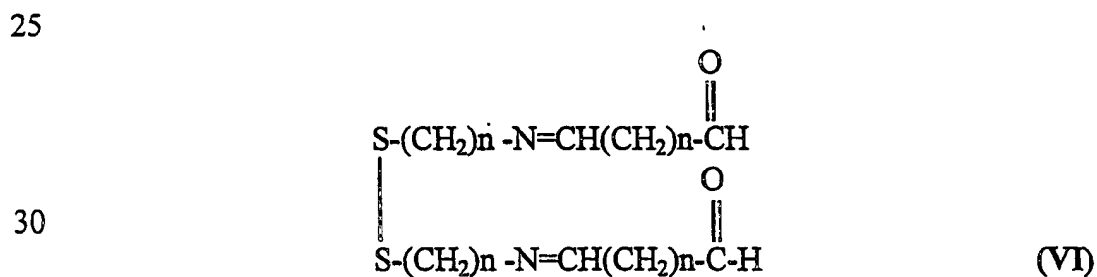
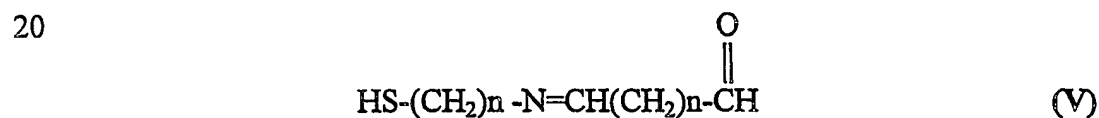
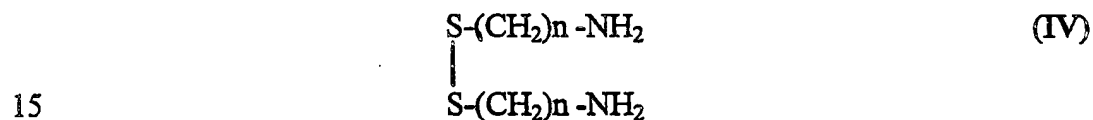
Q may for example be an amine group, capable of binding to a carboxyl residue; a carboxyl group, capable of binding to an amine residue; an isocyanate or isothiocyanate group or an acyl group capable of binding to an amine residue; or a halide group capable of binding to hydroxy residues of the binding entity. Particular examples are the groups $-\text{NH}_2$; $-\text{COOH}$; $-\text{N}=\text{C}=\text{S}$; $\text{N}=\text{C}=\text{O}$; or an acyl group having the formula $-\text{R}^a-\text{CO}-\text{G}$ wherein G is hydrogen, a halogen such



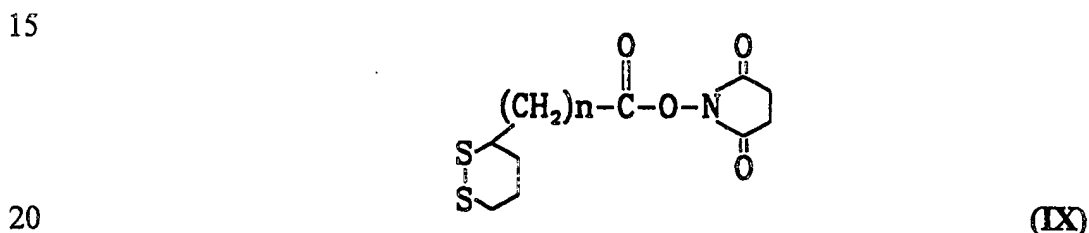
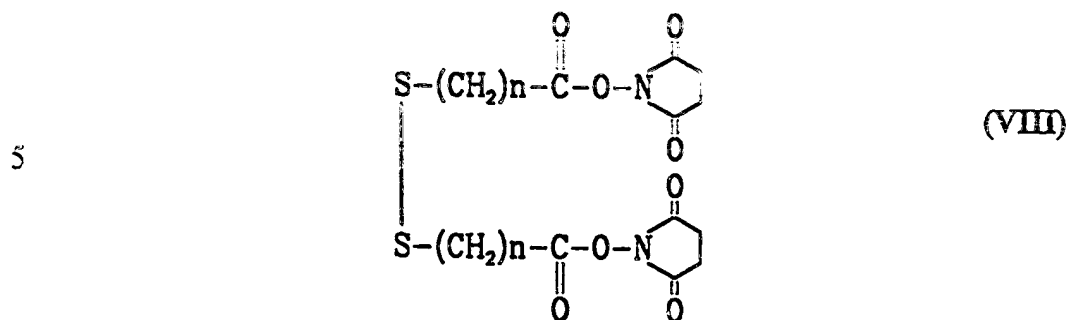
group; R^a and R^b being, independently a C_1 - C_{12} alkyl or alkenyl or a phenyl containing chain which is optionally substituted, e.g. by halogen.

5

Particular examples of such a linking group are those of the following formulae (III) - (IX):



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wherein n is an integer between 1-6.

As a result of binding of the cells onto the sensor or the release of such cells therefrom and the respective formation or dissociation of binding entities - cell complexes immobilized on the sensor, there results a change in total mass of the sensor which in turn results in a change of resonance frequency. This change thus provides an indication of the presence of said cells in the medium. The degree of change in the resonance frequency correlates with the extent of binding or release of said cells to or from the immobilized member and is dependent upon the concentration of said cells in the medium to which the sensor is exposed. Thus, the extent of change in the resonance frequencies may be used, in accordance with the invention, as an indication of the concentration of said cells in the medium.

In order to increase the sensitivity, use may be made with sensitivity increasing agents being auxiliary specific binding agents, which are free agents (i.e. *a priori* non-bound) that can specifically bind to an epitope on

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the surface of said cells. In accordance with this embodiment of the invention, after contact of the sensing member with said specimen, the sensing member is contacted with said sensitivity increasing agents which once bound to the cells increase the immobilized mass on the surface of the
5 sensing member and hence increases the change in the resonance frequency. Such sensitivity increasing agents may also be conjugated or complexed to large molecules or molecular complexes to further increase the change of mass. In addition, secondary auxiliary sensitivity increasing agents may be provided which specifically bind to the first auxiliary sensitivity increasing
10 agents already bound to the cell, thus bringing to an additional mass increase.

It is also possible in accordance with the invention to use a single sensor to determine the presence and optionally concentration of more than one type of cell in a medium. In accordance with this multiplexing sensor embodiment of the invention, the sensor is coated by a layer
15 comprising more than one specific binding entity, each one being capable of a specific binding to one of the cells to be assayed. When the specimen is contacted with such sensing member, all cells for which specific binding entities are immobilized on the sensing member, become bound thereto. In order to identify the cells bound to the sensing member, the sensing member
20 is first contacted with a first cell-specific agent which specifically binds to epitopes on the surface of such cells, and then a change in mass after such contact, measured by a change in the resonance frequency, is an indication that the respective cell is immobilized on the sensing member, and hence existed in the specimen. In order to verify then the existence of a second type
25 of cell, a second cell-specific binding agent is then contacted with the sensing member and change of resonance frequency serves then as an indication of the presence of the second respective cell in the specimen.

In order to increase the sensitivity of measurements performed in accordance with the multiplexing embodiment, use of the sensitivity increasing complexes, as described above, may be made.

The assayed liquid medium may for example be urine, vaginal
5 mucous, serum, plasma, whole blood, CSF, or generally any body fluid, Cells which may be assayed include yeast, e.g. candida; bacteria, e.g. chlamydia, mycoplasma, nicheria monocytogenes and others; as well as viral particles, e.g. HIV.

The invention will now be illustrated by a description of some
10 non-limiting specific embodiments, with occasional reference to the annexed drawings.

DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the sequence of steps in the preparation of a gold (Au)
15 plated piezoelectric crystal to immobilize anti-chlamydia antibodies (Abs) thereto to form a sensor member in accordance with the invention and subsequent use of the sensor for assaying of chlamydia in a medium;

Fig. 2 shows time-dependent changes in resonance frequency of
sensors, at different concentrations of *C.trachomatis* injected to the assay cell
20 suspended in urine, with the specific binding entity being a mouse-IgG-anti-*C.trachomatis* monoclonal antibody ("*C.trachomatis* binding entity"):

Fig. 2A shows results from a sensor where the *C.trachomatis* binding
entity is bound to Fc-specific, goat-IgG-anti-mouse IgG-Ab immobilized on
25 the sensor;

Fig. 2B. shows results from a sensor where the *C.trachomatis* specific
binding entity is bound to an immobilized goat IgG anti-mouse IgG, F(ab')₂.

In each case the concentration of *C.trachomatis* are as follows:

(a) 0.078 µg/ml; (b) 0.26 µg/ml; (c) 0.78 µg/ml; (d) 2.6 µg/ml; (e) 7.8 µg/ml;

Fig. 3 illustrates a manner of increasing sensitivity in the determination of chlamydia in a medium;

Fig. 4 shows additive changes in resonance frequency of a *C.trachomatis*-functionalized sensor as a function of time, upon treatment with different antibodies: (a) goat-IgG-anti-mouse IgG (Fc-specific); (b) mouse IgE anti-dinitrophenol; (c) mouse-IgG-anti-dinitrophenol; (d) mouse IgG anti-*C.trachomatis* P60; (e) mouse IgG anti-*C. trachomatis*-MOMP (major outer membrane protein); and (f) mouse-IgG anti-*C. trachomatis* LPS.

Fig. 5 shows sequential changes in resonance frequency of a quartz crystal functionalized for detecting *C.trachomatis* Ab, as a function of time upon interaction with *C.trachomatis* and subsequently with anti-*C.trachomatis* LPS: (a) *C.trachomatis*, 2.6 µg/ml; (b) anti-*C. trachomatis*, 0.175 µg/ml.

Fig. 6 Shows time-dependent changes in resonance frequency of a quartz crystal with a sensing interface having immobilized anti-*C.trachomatis* LPS-Ab attached to goat IgG anti-mouse-IgG, Fc-specific antibodies, pre-treated with 5 mg/ml BSA: (a) addition of a new BSA sample; (b) addition of *C.trachomatis*, 0.26 µg/ml; (c) subsequent to exposure to chlamydial cells as in (b), exposure to anti-*C.trachomatis* LPS-Ab. 0.175 µg/ml.

Fig. 7 shows time-dependent changes in resonance frequency of a functionalized quartz crystal pre-exposed to urine upon exposure to fresh urine specimens that include variable concentrations of *C.trachomatis*: (a) 0.078 µg/ml; (b) 0.26 µg/ml; (c) 0.78 µg/ml; (d) 2.6 µg/m.

Fig. 8 shows time-dependent resonance frequency changes of a functionalized crystal, pre-exposed to urine, upon interaction with fresh urine samples that include variable concentrations of *C.trachomatis* and subsequent amplification/confirmation of the primary analysis: (a) exposure of the sensor with *C.trachomatis* in urine, 0.26 µg/ml; (b) subsequent exposure of the

sensor of (a) with anti-*C. trachomatis* LPS-Ab, 175 µg/ml in PBS; (c) exposure of the sensor to *C.trachomatis*, 2.6 µg/ml in urine; (d) subsequent exposure of the sensor of (c) to anti-*C. trachomatis* LPS- Ab, 0.175 µg/ml in PBS; (e) exposure of the sensor to a sterile urine sample; (f) subsequent
5 treatment of the sensor of (e) with the anti-*C. trachomatis* LPS-Ab, 0.175 µg/ml in PBS.

Fig. 9 shows the effect of storage time of the sensors on the resonance frequency changes following exposure to 0.26 µg/ml. The electrodes were functionalized by mobilization of anti-*C.trachomatis* antibodies by two
10 different routes: by goat-IgG-anti-mouse-IgG antibodies (filled circles); and by fragmented F(ab')₂ anti-mouse IgG-antibodies (filled squares).

DETAILED DESCRIPTION OF THE INVENTION

Experimental setup and procedures:

15 Quartz crystals sandwiched between two gold (Au)-electrodes, are the preferred sensors of the present invention. All measurements described below were performed using 9 MHz quartz piezocrystals (QPC) (AT cut type) covered with a layer (ca. 0.2 cm²) consisting of sputtered gold (ca. 3000Å) on a titanium (Ti) substrate (ca. 500 Å) (Seiko EG&G). The frequency
20 measurements were performed using a Quartz Crystal Analyzer (model QCA917, Seiko EG&G) linked to a personal computer. To functionalize the sensor, first it is cleaned by treatment with HCl consisting of a first soaking for two minutes in 1 M HCl solution, then after rinsing and drying, applying 50 µl concentrated HCL on the gold surfaces for two minutes followed by
25 rinsing.

The sensing interfaces for sensing *C.trachomatis* may be prepared as schematically outlined in Fig. 1: a cystamine monolayer is assembled on the Au-electrodes associated with the quartz crystal by treatment of the crystal, in a test-tube, with a 0.02 M aqueous solution of

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cystamine dihydrochloride for two hours. The resulting electrode is then rinsed with ethanol and water. In the next step, the monolayer-modified sensing surfaces are reacted with a PBS (phosphate buffer saline) solution, (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂P0₄, 1.8 mM KH₂PO₄, pH=7.4) that includes 2 mM Sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate (sulfo-SMPB), for one hour; thus the sulfo-SMPB is covalently linked to the base cystamine monolayer. The surface densities of the cystamine sub-monolayer and sulfo-SMPB can be determined by following the crystal resonance frequencies at each modification step and the application of the Sauerbrey relation. The resulting functionalized crystal is further modified by linking cysteine residues of anti-mouse IgG Ab or of fragmented F(ab')₂ anti-mouse IgG Ab to the maleimide residues on the monolayer, by two alternative routes: (a) The electrodes are reacted with polyclonal goat IgG anti-mouse IgG, or (b) the electrode are reacted with fragmented polyclonal goat IgG anti-mouse IgG, F(ab')₂. These modifications are carried out by the interaction of the electrode with 17 µg/ml of the respective antibodies in 50 mM HEPES buffer solution that included 10 mM EDTA, pH=6.5, for 30 minutes. The resulting antibody functionalized interfaces are rinsed with PBS buffer solution and further treated with 1.7 µg/ml mouse-IgG-anti-*C. trachomatis* LPS (lipopoly-saccharide) antibody in PBS solution for 30 minutes, to yield the active sensing interface. The Fc fragment of the latter antibody binds to the IgG base layer. Monitoring and verifying the addition steps can be performed by following the equivalent frequency changes of the crystal.

The assembly of the layered sensing interface prepared as above was characterized experimentally by following the crystal resonance frequency changes after each modification step. Table 1 below summarizes typical values of the crystal frequency changes at the different steps of modification upon assembly of the anti-*C. trachomatis* Ab to the anti-mouse

IgG-Ab or the F(ab')₂ fragmented anti-mouse IgG-Ab. The surface coverage of the different components are also included in the Table. It should be noted that these frequency changes are only representative values and some deviations can be expected, especially for the primary two layers of cystamine and sulfo-SMPB, due to differences in the roughness of the Au-electrodes.

Table 1: typical resonance frequency changes (Δf) of quartz crystals upon stepwise assembly of the components in the sensing interfaces and the respective calcu-

components^{a)}.

Modification step	Sensing interface ^{b)}		Sensing Interface ^{c)}	
	Δf (Hz)	σ (mol·cm ⁻²)	Δf (Hz)	σ (mol·cm ⁻²)
Cystamine	-202	$7.562 \cdot 10^{-9}$	-195	$7.300 \cdot 10^{-9}$
Sulfo-SMPB	-105	$2.315 \cdot 10^{-9}$	-95	$2.095 \cdot 10^{-9}$
Untreated anti-mouse IgG Ab	-105	$4.0 \cdot 10^{-12}$	--	--
F(ab') ₂ anti-mouse IgG	--	--	-89	$5.1 \cdot 10^{-12}$
Anti- <i>C.trachomatis</i> Ab	-63	$2.4 \cdot 10^{-12}$	-74	$2.8 \cdot 10^{-12}$

^{a)} Calculated using Equation 1.

^{b)} Sensing interface consists of the goat IgG anti-mouse IgG, Fc-specific, Ab as sublayer.

^{c)} Sensing interface consists of the goat F(ab')₂ anti-mouse IgG Ab as sublayer.

For assaying, the resulting sensor units are mounted in an assay cell, and the cell is then ready to be filled with the test solution. The crystal is

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allowed to stabilize to a constant resonance frequency typically ± 1 Hz. Samples of *C. trachomatis* in PBS of variable concentrations are injected into the cell and the crystal frequency changes can be monitored as a function of time.

5

Different manner of immobilization of anti-*C.trachomatis* antibodies on the sensors:

C. trachomatis samples suspended in urine were injected into the assay cell. The *C.trachomatis*-modified crystals were then rinsed with the PBS buffer, 10 ml, 2 ml/min. Fig. 2 shows the frequency changes of crystals tailored by the two methods upon interaction of the electrode with different concentrations of *C.trachomatis*. In these experiments, the electrodes are consecutively treated with increased concentrations of *C. trachomatis*. Fig. 2A shows the resonance frequency changes of a sensor having anti-*C.trachomatis* antibody immobilized onto the whole anti-mouse IgG antibody layer. Fig. 2B shows the resonance frequency changes of a sensor having anti-*C.trachomatis* sensing layer immobilized onto a fragmented F(ab')₂ anti-mouse IgG layer. In both systems, as the concentration of the bacteria increases, the frequency of the crystal decreases, implying the association of *C.trachomatis* to the sensing interface. For example, for the sensor with immobilized anti-*C.trachomatis* antibodies, in the presence of bacteria at a concentration of 0.26 and 2.6 $\mu\text{g/ml}$, the crystal frequency changes after 350 secs. is $\Delta f = -6$ Hz and $\Delta f = -34$ Hz, respectively (Fig. 2A); for the sensor with immobilized fragmented anti-*C.trachomatis* antibodies, the corresponding values are $\Delta f = -5$ Hz and $\Delta f = -32$ Hz, respectively (Fig. 2B). The response-time for the detection of the bacteria is ca. 350 sec. and is defined as the time after which the crystal frequency levels off at low concentrations of *C.trachomatis*.

Means for increasing sensitivity of the invention without sacrificing specificity:

5 In order to increase the sensitivity of the sensor, both with respect to a minimal amount of cells to be assayed, as well as to improve the sensor's differential response to different concentrations of the assayed cells, additional steps can be implemented. This may be achieved by utilizing free specific binding agents, e.g. specific anti-cell antibodies as illustrated in
10 Fig. 3. As shown in Fig. 3, after a step allowing binding of cells, *C.trachomatis* in the specific example, the sensor is exposed to free specific binding agents, which are free anti-*C.trachomatis* LPS antibodies in the specific example, and following such exposure, such antibodies bind to the cell and increase the immobilized mass. The sensitivity increase conferred by
15 such antibodies (hereinafter: "*sensitivity increasing antibodies*"), can further be increased by secondary sensitivity increasing antibodies which bind to the first sensitivity increasing antibodies. Such increase in sensitivity is demonstrated in the following experiment.

 The anti-*C. trachomatis* antibodies [anti-LPS, anti-P60 or
20 anti-MOMP (major outer membrane protein)] were injected into the assay cell, at 0.175 µg/ml, and the crystal's resonance frequency changes were followed as a function of time. The anti-*C. trachomatis* functionalized electrodes were treated, after rinsing, with the polyclonal, Fc-specific, goat IgG-anti-mouse IgG, 0.175 µg/ml, to obtain further amplification. The crystal
25 frequencies were monitored as a function of time to follow the association of the antibodies.

 Fig. 4 shows the resonance frequency changes of the sensor after exposure to *C.trachomatis*, 2.6 µg/ml, followed by treatment with a variety of potential sensitivity increasing antibodies: goat anti-mouse IgG
30 (curve a), mouse IgE anti-dinitrophenol (curve b) and mouse IgG

anti-dinitrophenol (curve c). The crystal frequency changes of the *C. trachomatis*-modified surface in the presence of three different anti-*Chlamydia* monoclonal antibodies, are shown: anti-P60 (60 kD membrane protein) (curve d), anti-MOMP (curve e), and anti-*C. trachomatis* LPS (lipopolysaccharide) (curve f). With the unrelated IgG and IgE, anti-dinitrophenol antibodies and the anti-mouse IgG antibody, no frequency changes were observed (curves a, b and c). These latter control experiments clearly indicate that the base layer of the anti-mouse IgG is saturated with the anti-*C. trachomatis* Ab and no foreign IgG binds to the surface. The secondary anti-*C. trachomatis* antibodies caused a frequency decrease of the crystal, meaning that these antibodies bind to the immobilized *C. trachomatis* cells. Thus, the interaction of the antibodies with the sensing interface that was previously exposed to *C. trachomatis* provides a confirmatory test for the initial association of the cells to the monolayer.

Fig. 5 shows the resonance frequency changes of the sensor pre-exposed to the bacteria upon exposure to anti-*C. trachomatis* LPS antibodies (curve a), and after subsequent exposure to goat anti-mouse IgG antibodies (curve b). Exposure of the sensor to the anti-LPS antibodies results in a resonance frequency change of $\Delta f = -16$ Hz. The subsequent exposure yields an additional decrease in the crystal's resonance frequency (curve b) of $\Delta f = -18$ Hz, implying that this secondary sensitivity increasing antibody binds to the anti-LPS. These results clearly indicate that the anti-LPS, being an IgG antibody, does not associate to a defective sublayer site but interacts with the bacteria antigen. This interaction yields the exposed Fc site of the IgG-Ab that permits the secondary binding of goat anti-mouse IgG-Ab. This succession provides an added advantage in that in addition to amplification of the response of the initial binding of *C. trachomatis* to the sensing interface, the cumulative frequency decrease observed provides also a built in continuation of the assay structure of the sensing mechanism.

Sensitivity towards non chlamydial protein:

Although the anti-*C.trachomatis* sensing interface is unaffected by foreign proteins such as cytochrome oxidase, cytochrome C or glucose oxidase, it was found that there may occur some non-specific binding to the sensing surface by proteins such as BSA. Interaction of the electrode with BSA at 5 $\mu\text{g/ml}$ results in a frequency change of ca. -100 Hz due to non-specific adsorption of the protein to the surface. The BSA-treated sensing surface is, however, not further influenced upon interaction with BSA, but reveals the original affinity for *C.trachomatis*. Fig. 6 shows the frequency changes of the crystal pre-treated with BSA upon interaction with a new BSA sample (curve a) and upon treatment with *C.trachomatis* 0.26 $\mu\text{g/ml}$. The BSA does not influence the crystal frequency, but the antigen-bacteria results in a frequency decrease, $\Delta f = -6$ Hz, implying the association of the analyte antigen to the surface. Thus, the treatment of the sensing interface with BSA results in the non-specific adsorption of the protein, but blocks a further non-specific adsorption phenomena. The BSA-blocked interface, however, retains its affinity for sensing *C.trachomatis*.

As an alternative manufacturing technique for specific sensing interfaces, such non-specific adsorption is impeded by introducing an extra step within the process of preparing of the electrodes: the anti-*C. trachomatis* LPS Ab is associated to the bare antibody layer in the presence of BSA at 5 mg/ml (see Fig. 1). The resulting sensing interface is unaffected in the presence of BSA but reveals analogous activity for sensing *C. trachomatis*. Fig. 6 (curve c) shows the resonance frequency changes of a BSA pre-treated electrode after treatment with *C.trachomatis* at 0.26 $\mu\text{g/ml}$ and subsequent incubation with the anti-*C.trachomatis* LPS Ab. The observed frequency decrease confirms the primary association of the antigen-bacteria to the sensing interface and amplifies the transduced signal.

Measurements in urine environment:

A preferred embodiment of the invention is aimed at detecting the chlamydia in urine samples. The performance of the sensor in a real urine sample was examined. The BSA-treated sensors were treated with *C.trachomatis* free urine. This results in a frequency change of the crystal of ca. -50 Hz due to the non-specific association of the urine ingredients. The resulting probes were insensitive towards additional pure urine specimens but revealed activities in sensing the specific cells in urine samples. Inactivated *C.trachomatis* bacteria were dispersed in urine and the crystal was challenged with different cell concentrations in urine. Fig. 7 shows the responses of the sensor in the presence of different samples of urine containing variable concentrations of Chlamydia cells. The crystal frequency decreased as the concentration of the *C.trachomatis* in the analyte sample increases. It is noted, however, that the extent of frequency decrease is substantially lower than the values observed when the antigen was dissolved in a pure PBS buffer solution. For example, in the presence of *C.trachomatis* at concentrations corresponding to 0.26 and 0.78 mg/ml, the frequencies changes of the electrode in urine are -1 and -9 Hz, whereas in PBS solutions, the frequency changes are -6 and -17 Hz, respectively [cf. Fig. 3(A)]. This implies that the sensitivity of the probe decreases in the presence of urine.

The decrease in the frequency responses of the crystal in the presence of urine could be attributed to viscosity changes of the urine solution which opposes the frequency changes induced by the mass changes occurring on the crystal. To overcome this difficulty, and to retain the sensitivities observed in the PBS buffer solution, the confirmatory test that involves the free antigen-LPS Abs and the measurement performed with urine were combined. In this experiment, the sensing interface was interacted with *C.trachomatis* containing urine to yield the low frequency change as

discussed above. After association of the antigen bacteria to the sensing interface, the cell was rinsed with PBS buffer. The anti-LPS Ab was then injected to the cell and the high-value frequency change as a result of the association of the antibody to *C.trachomatis* in PBS was observed. Fig. 8 shows the crystal frequency changes upon interaction of the sensing interface with urine containing *C.trachomatis* at concentrations corresponding to 0.26 $\mu\text{g/ml}$ and 2.6 $\mu\text{g/ml}$ (curves a and c, respectively). With the sample containing 0.26 $\mu\text{g/ml}$ of the antigen-bacteria, a very low frequency change, $\Delta f = -2$ Hz, was observed. Upon rinsing the assay cell with the PBS buffer solution and treatment of the electrode with the anti-LPS antibody, high frequency changes of the electrode were observed. For the systems that were interacted with urine containing *C.trachomatis*, 0.26 and 2.6 $\mu\text{g/ml}$ frequency changes of $\Delta f = -5$ Hz (curve b) and -15 Hz (curve d), respectively were observed after rinsing with PBS buffer and interaction with anti-LPS Ab, respectively. Fig. 8 (curve e) shows the response of the sensing interface upon exposure to a *C.trachomatis*-free urine, and curve f shows the response of the electrode after rinsing with the PBS buffer solution and injection of the anti-LPS Ab. No frequency changes in the electrode response are observed consistent with the fact that no bacteria were present in the analyzed sample.

Thus, the recommended protocol for the analysis of *C.trachomatis* involves the interaction of the sensing interface with the analyte urine sample followed by rinsing of the assay cell with a PBS buffer solution and then introduction of the anti-LPS antibodies. A resonance frequency change of ca $\Delta f = -5$ Hz in the second step should be considered as a positive indication for the presence of the antigen bacteria in the urine sample at a concentration of ca. 0.26 $\mu\text{g/ml}$.

Stability of the sensors under storage conditions:

The performance of the two types of immobilization of the anti-*C.trachomatis* to the sensing interfaces, namely, linkage of the antibody to the goat anti-mouse IgG antibody, or association to the f(ab')₂-fragmented goat anti-mouse IgG antibody (cf. Fig. 1), were examined as a function of storage-time. The electrodes were stored in sealed vials at 4°C for different denotations. Fig. 9 shows the resonance frequency changes of the sensors in the presence of *C.trachomatis* 0.26 µg/ml, as a function of the sensors' durations of storage. The sensors having a base layer of whole goat anti-mouse IgG antibodies exhibit a degradation with time, whereas the electrodes where the base antibodies are fragmented F(ab')₂ goat anti-mouse IgG antibody reveal unaltered activity for a period of 90 days of storage.

Mixed type probes:

This specific probe may be used in cases where more than one microorganism may be a potential cause of a given infection. Such a probe carries on its surface a plurality of specific binding entities, each one specific for one of the cells to be assayed, e.g. one entity being a specific for *Chlamydia* and the other being specific for *N. Gonorrhoeae*. A urine specimen, by a specific example, is then contacted with the probe. If both types of cells exist in a specimen, then both such cells will become immobilized on the probe. This will give rise to a change of mass, and by measuring the Δf response indication of binding of cells to the probe will be provided. Then there remains the problem of identifying which of the cells exists in the sample, i.e. whether only *E. Coli*, or only *Chlamydia* or both. This may first be achieved for example by a sequential exposure of the probe first to a free specific binding agent which specifically binds to one of such cells and then to a second free specific binding agent which specifically binds to the other of such cells. Such agent may for example be a monoclonal

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antibody or lectin. A result of binding of such an agent to the cells, there is an increase in mass which is then measured by the resulting Δf response. Obviously, the sensitivity of such a measurement can be increased by complexing such an agent either prior to contact with the probe or after
5 allowing binding, with a large molecule or a complex of molecules which thus induces a more pronounced mass change.

At the next step, the probe may be contacted with an agent which specifically binds to the other cell and then a mass change is measured in a similar manner as that described above.

10 If the first step in the above sequence does not give rise to change in mass this may serve as an indication that the cells which became immobilized on the probe were the second type of cells. If there is a change of mass only after the first step in the above sequence and not after the second, this is an indication that only the first type of cells in the specimen. If
15 there is a change of mass after both steps in the sequence, this is an indication that both cells existed in the specimen.

CLAIMS:

1. A system for assaying cells in a liquid medium, the system comprising a piezoelectric crystal-based sensing member; an electric or
5 electronic unit electrically connectible to the crystal for generating a vibration inducing electric current causing vibrations in said crystal and for measuring its resonance frequency; and a vessel for holding a specimen of said liquid medium and allowing contact thereof with said sensing member; the sensing member comprising a piezoelectric crystal provided with one or more metal
10 plates on its surface, the metal plates having immobilized thereon specific binding entities, which specifically bind to an epitope on the surface of said cells, such that the binding of the cells to the immobilized specific binding entities or release of said cells from the immobilized member bringing to a change of mass of the sensing member, resulting in a change of the sensing
15 member's resonance frequency measurable by said unit.
2. A system according to Claim 1, wherein non-specific protein binding sites on the surface of the sensing member are blocked by a protein adsorbed thereto.
3. A system according to Claim 2, wherein said protein is bovine
20 serum albumin.
4. A system according to any one of Claims 1-3, said cells are chlamydia and said aqueous medium is urine.
5. A system according to any one of Claims 1-3, wherein said specific binding entities comprise binding entities specific for two or more
25 types of cells.
6. A system according to any one of Claims 1-5, wherein said specific binding entities are antibodies or fragments thereof which retain the binding specificity of the antibodies.

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7. A system according to Claim 6, wherein said antibodies or fragments are immobilized by means of additional antibodies which specifically bind said antibodies or fragments, immobilized on the surface of said metal plates.

5 8. A system according to any one of Claims 1-7, comprising a sensitivity increasing agent, being free agents which specifically bind to said cells, which are added into said vessel after contact of the sensing member with the assayed specimen.

9. A method for assaying presence and optionally concentration of
10 cells in a liquid medium, the method comprising:

(a) providing a sensing member comprising a piezoelectric crystal with one or more metal plates on its surface, the metal plates having immobilized thereon specific binding entities which specifically bind to epitopes on the surface of said cells;

15 (b) contacting said sensing member with a specimen of said medium for a time and under conditions allowing binding of the cells to said specific binding entities;

(c) inducing vibrations in said crystal and measuring a change in the resonance frequency as a result of either

20 (ca) contact of said sensing member with said specimen,

(cb) contact as in (ca) and then incubation of said sensing member under conditions and for a time such that cells bound to said specific binding entities will be released, or

(cc) contact as in (ca) followed by exposure of the sensing
25 member to a sensitivity increasing agent, which is a free agent which specifically binds to an epitope on the surface of said cells; said change being an indication of presence of said cells in said medium.

10. A method according to Claim 10, wherein the extent of said change serves as an indication of the concentration of said cells in said medium.

11. A method according to Claim 9 or 10, wherein said cells are
5 chlamydia and said liquid medium is urine.

12. A method according to any one of Claims 9-11, wherein said specific binding entities comprise two or more binding entities, each specific for a different cell.

13. A sensing member for use in assaying presence of cells in a
10 liquid medium comprising a piezoelectric crystal electrically connectible to a unit for inducing vibrations in the crystal and for measuring its resonance frequency, the piezoelectric crystal provided with one or more metal plates on its surface, the metal plates having immobilized thereon specific binding entities, which specifically bind to an epitope on the surface of said cells, such
15 that the binding of the cells to the immobilized specific binding entities or release of said cells from the immobilized member bringing to a change of mass of the sensing member, resulting in a change of the sensing member's resonance frequency measurable by said unit.

14. A sensing member according to Claim 13, wherein non-specific
20 binding sites on said metal plates are blocked by proteins absorbed thereto.

15. A sensing member according to Claim 14, wherein said protein is serum albumin.

16. A sensing member according to any one of Claims 13-15, wherein said specific binding entities specifically bind to an epitope on the
25 surface of chlamydia cells.

17. A sensing member according to any one of Claims 13-15, wherein said specific binding entities comprise binding entities specific for two or more types of cells.

18. A sensing member according to any one of Claims 13-17, wherein said specific binding entities are antibodies or fragments thereof which retain the binding specificity of the antibodies.

19. A sensing member according to Claim 18, wherein said
5 antibodies or fragments are immobilized by means of additional antibodies which specifically bind said antibodies or fragments, immobilized on the surface of said metal plates.

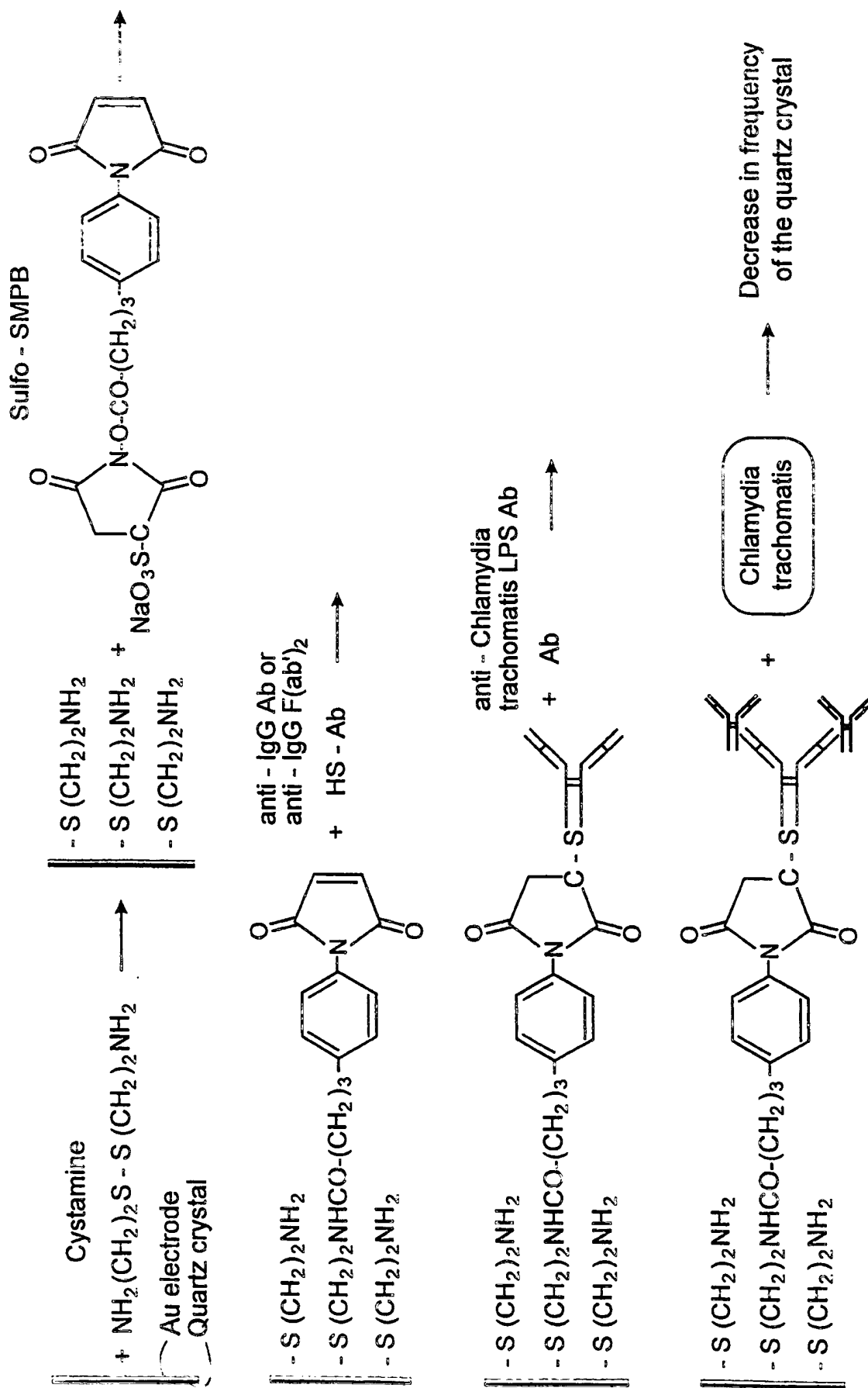


Fig. 1

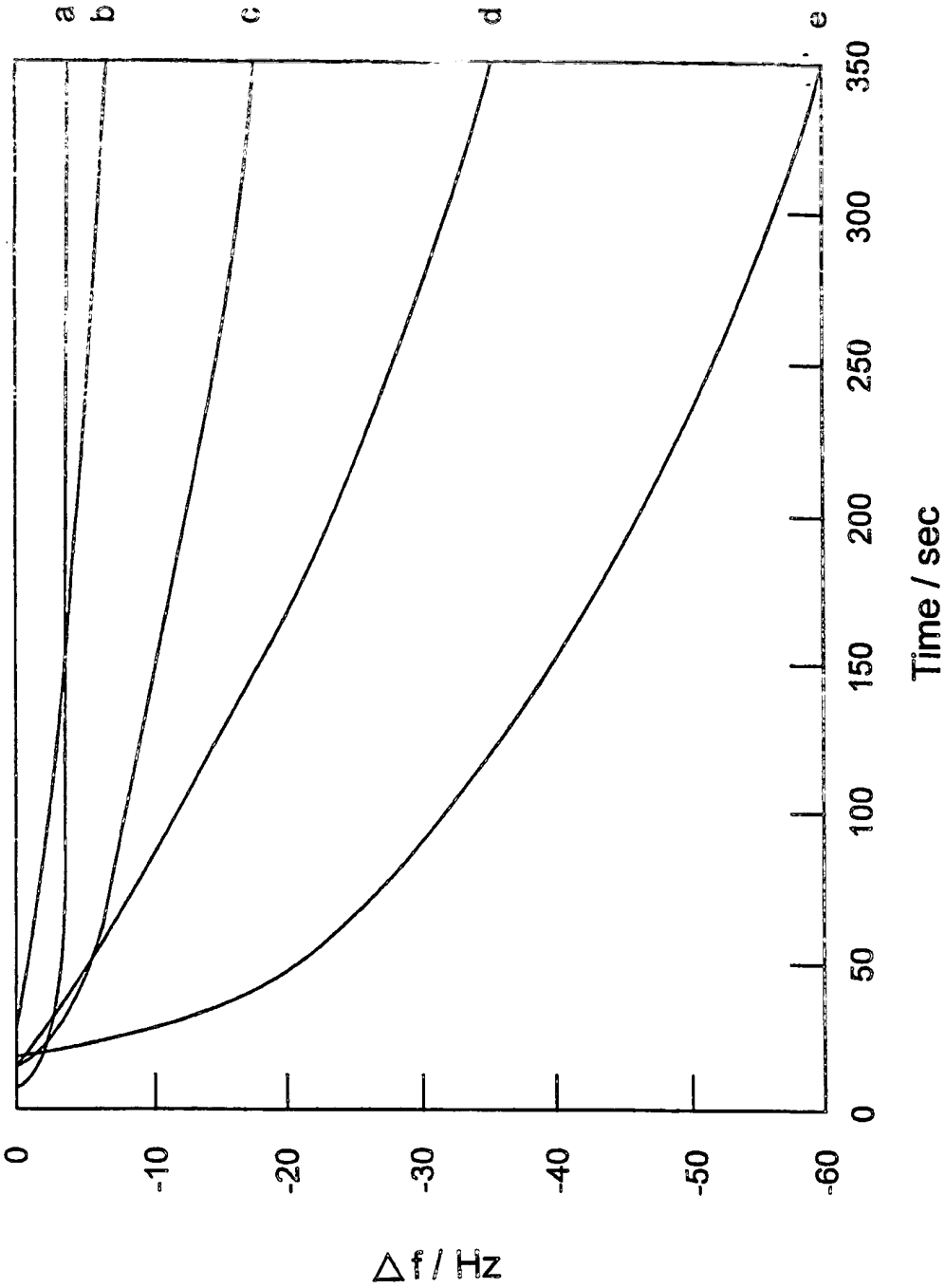


Fig. 2A

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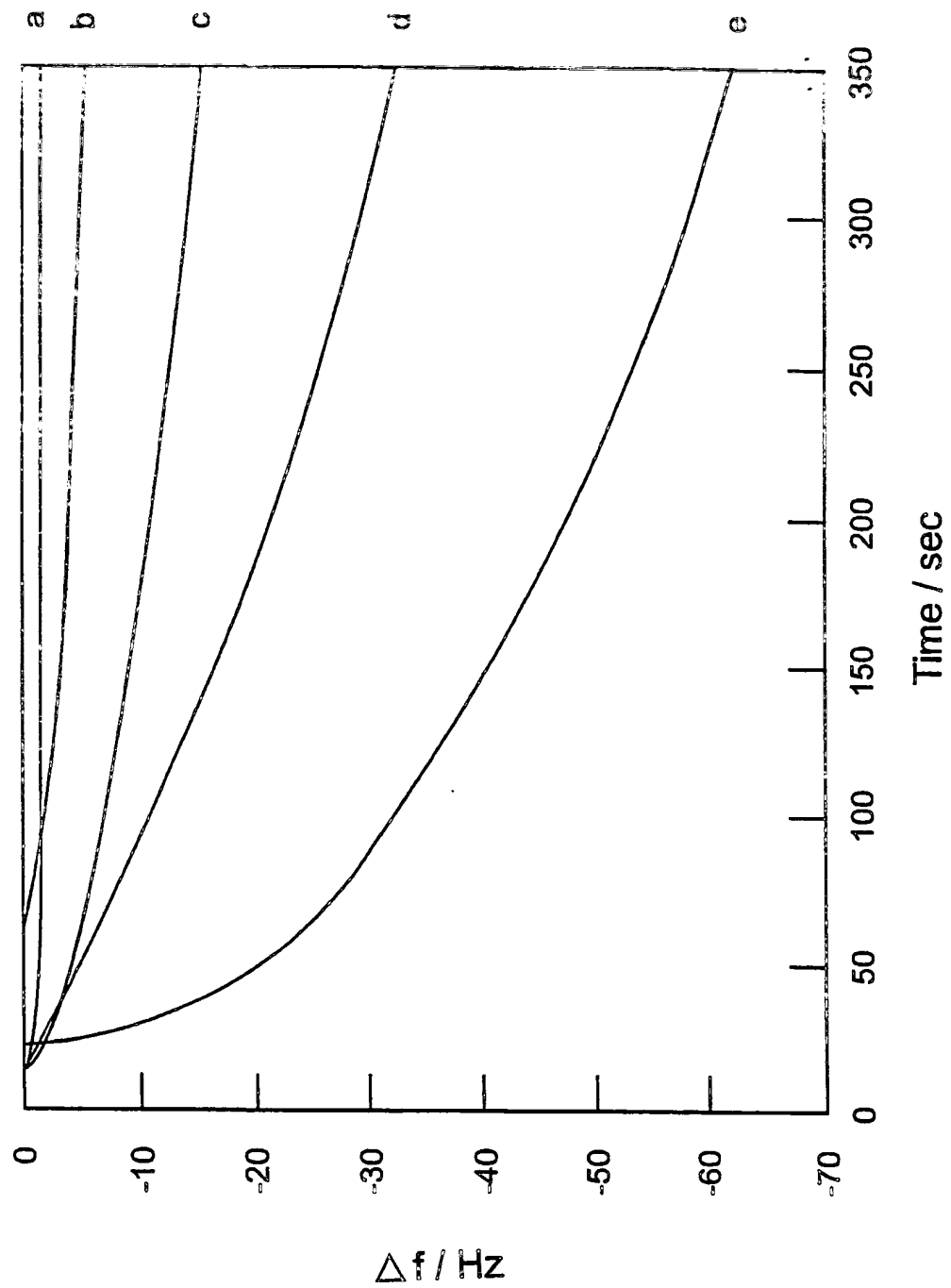


Fig. 2B

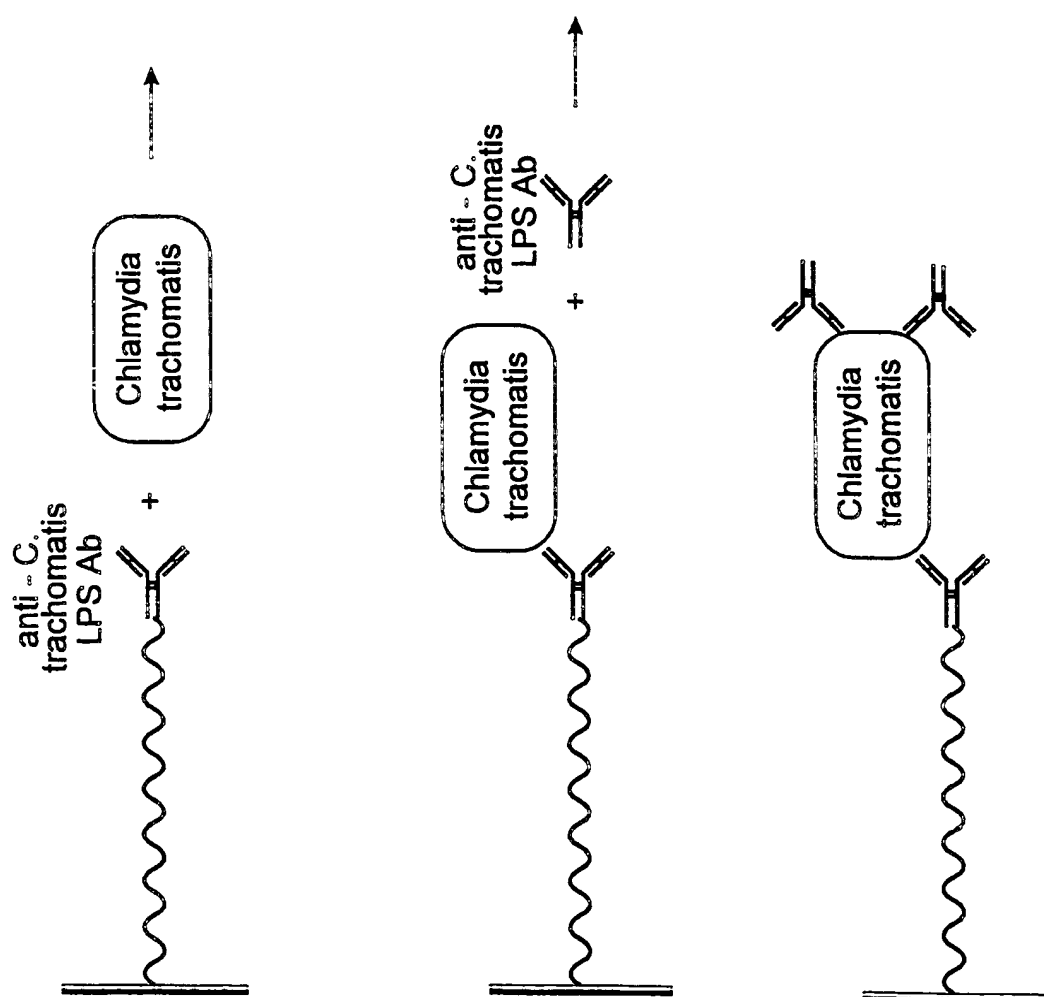


Fig. 3

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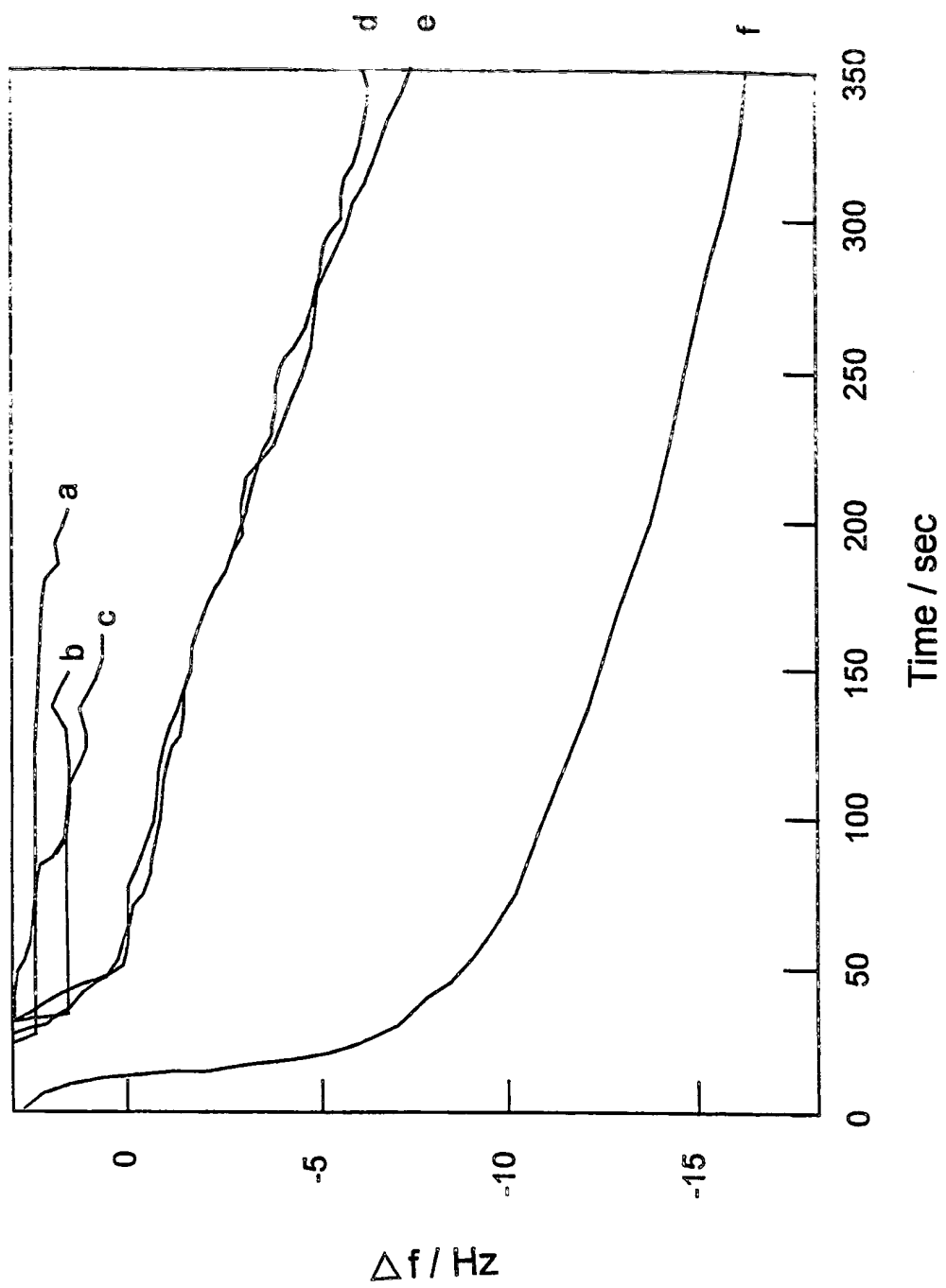


Fig. 4

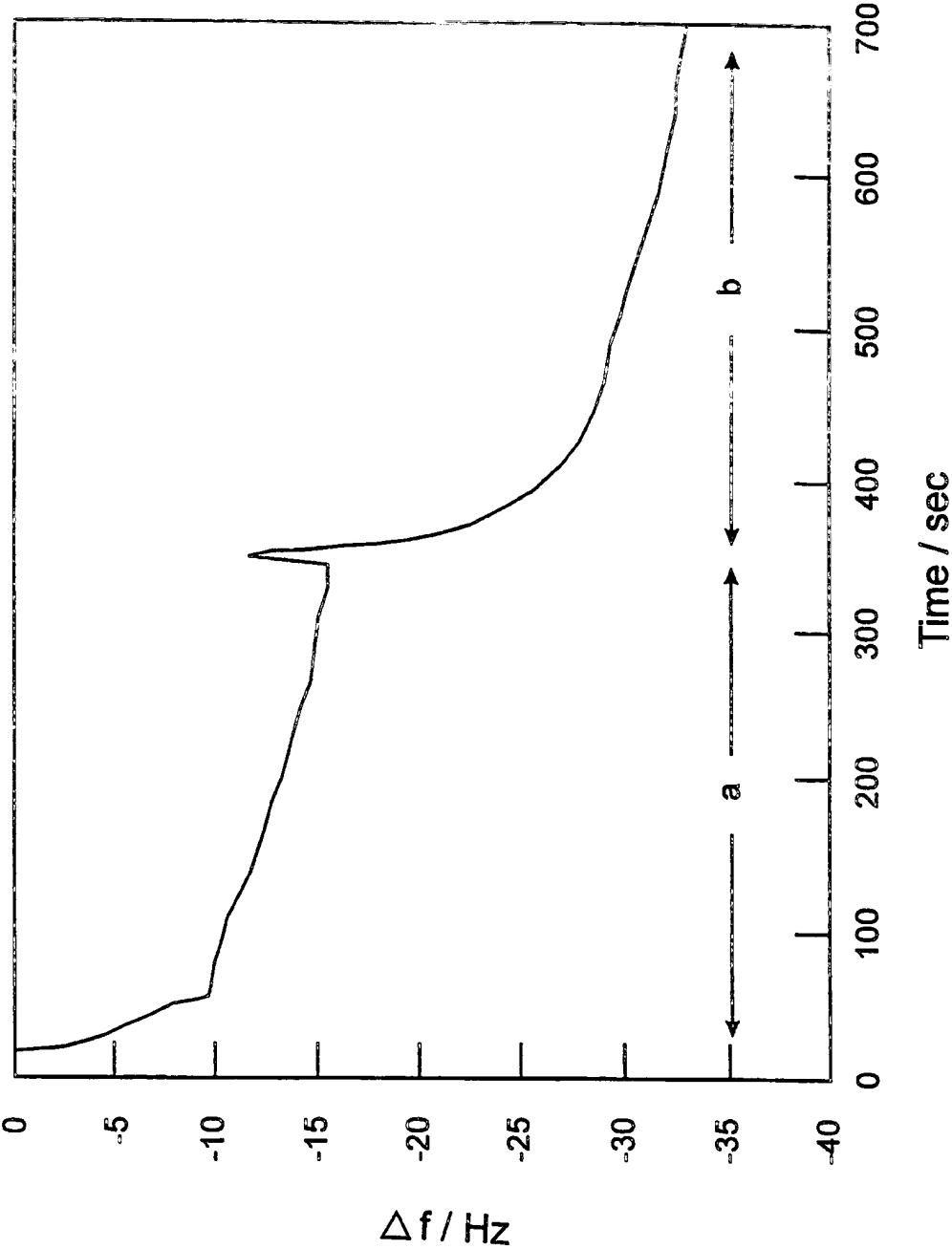


Fig. 5

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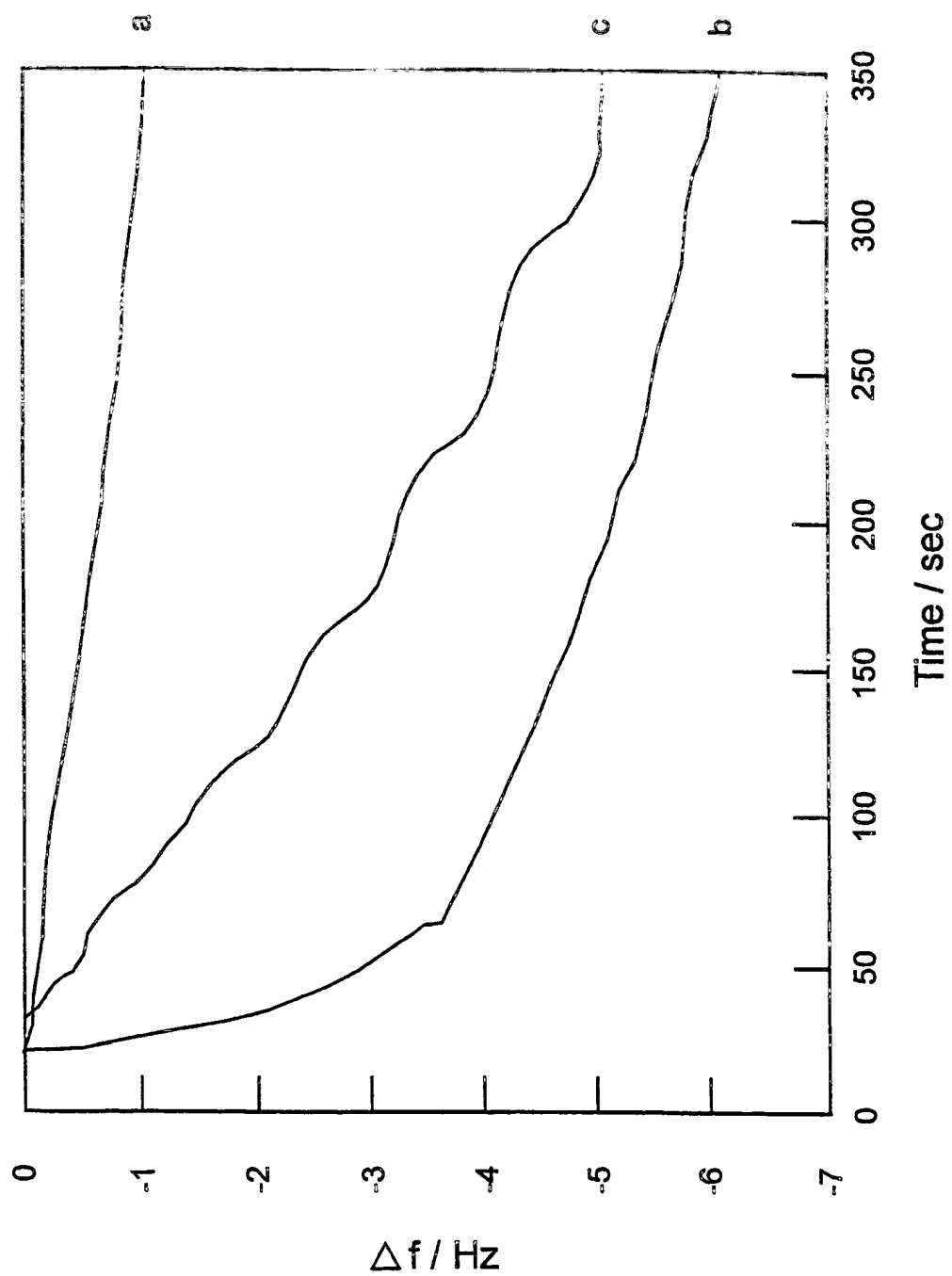


Fig. 6

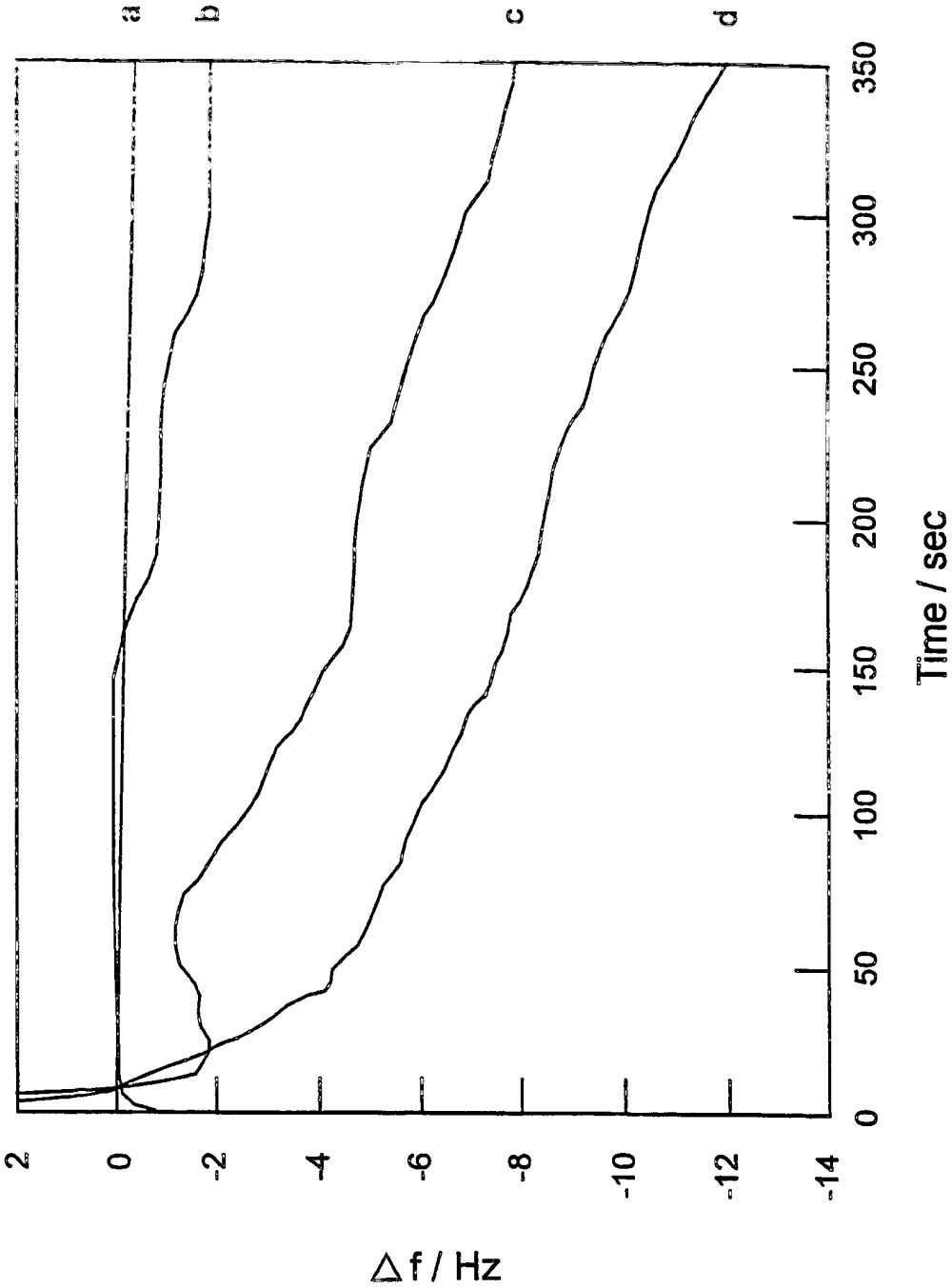


Fig. 7

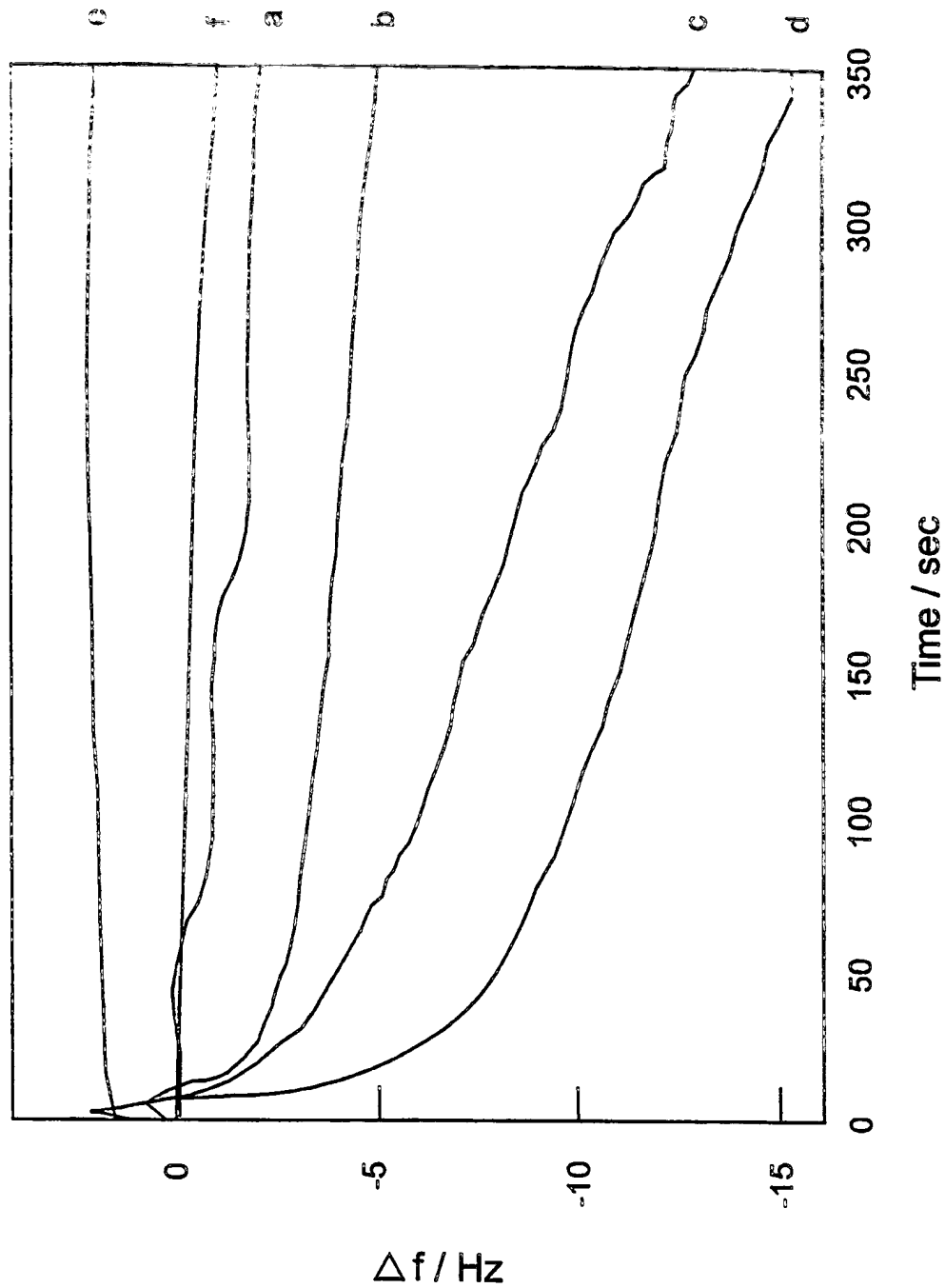


Fig. 8

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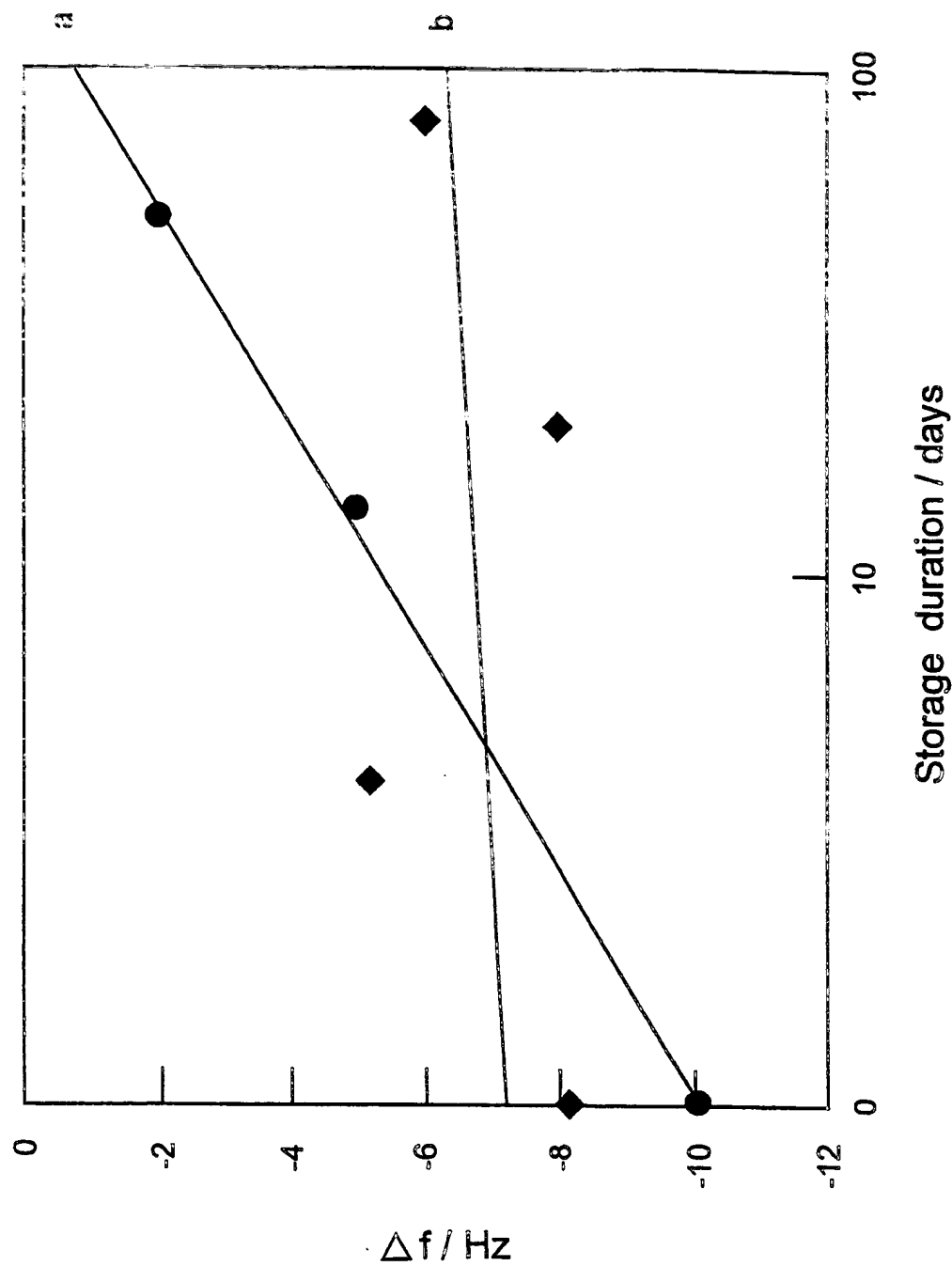


Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/IL 98/00119

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N27/327

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"Z" document member of the same patent family

Date of the actual completion of the international search

28 July 1998

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.O. Box 5310 Patentplan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tlx. 31 651 cpa n
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Authorized officer

Moreno, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IL 98/00119

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International Application No.

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C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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International Application No.

PCT/IL 98/00119

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